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In vivo assessment of the oxidative and apoptotic potential of perfluorooctanoic acid

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ABSTRACT

Perfluorooctanoic acid (PFOA) is an organic pollutant abundant in the environment, and the potential toxicity of which is causing great concern. The oxidative and apoptotic potential of PFOA was assessed in a short-term in vivo zebrafish assay in this study. Activities of antioxidative enzymes, including superoxide dismutase and catalase, were significantly altered in PFOA-treated embryos. Lipid peroxidation was increased with exposure to higher concentrations. Expression profiles of certain genes related to cellular apoptosis were then investigated. The expression of p53 and Baxwere partially increased, which could be linked to PFOA-induced cell apoptosis in zebrafish. The overall results demonstrated that PFOA could induce oxidative stress and alter the expression of genes involve in apoptosis, resulting in oxidative damagesin zebrafish embryos.

KEYWORDS

Oxidative stress;
Perfluorooctanoic acid;
Zebrafish.

INTRODUCTION

Perfluorooctanoic acid (PFOA) is a perfluorinated compound (PFC), which consists of a carbon backbone and hydrogen replaced by fluorine. The strong carbon-fluorine bonds result in remarkable chemical stability and the compound therefore do not undergo photolysis, hydrolysis, or biodegradation. Over the recent years, numerous reports of increasing concentrations of PFOA in the environment such as air, water and soil, as well as in wildlife and humans have been published[2,5,14,25]. PFOA is well absorbed but poorly cleared with a half-life of 3.8 years in humans[11]. It is well established that human serum PFOA levels are elevated in communities with highly contaminated drinking water[14].

PFOA may cause health effects in animals and humans. Information on the developmental toxicity of PFOA in rodents and birds have been reported and illustrate the developmental toxicity, hepatotoxicity, reproductive toxicity, immunotoxicity and carcinogenicity of PFOA in experimental animals[4,6,19]. A genomic analysis demonstrates that genes involved in the transport and metabolism of fatty acids and lipids, cell communication, adhesion, growth, and apoptosis are significantly altered in PFOA treated rats[16].

Aquatic ecosystems serve as the ultimate sink for many environmental pollutants that accumulate in fish species. The average concentrations of PFC in the fish can reach 8850-fold greater than those in surface wa-

ter[9]. Toxicant exposures that cause oxidative stress during embryonic development are a significant health risk in adults. Fish embryonic development may be especially sensitive to toxicant-induced oxidative stress since even a 15–20% increase in ROS can tip progenitor cells into premature cell cycle arrest or differentiation[21]. As a result of this oxidative stress, fish, like many other vertebrates, try to reduce the damage using the antioxidant defense system. While oxidative effects of PFOA are well documented in rodents, the information in fish is scarce and variable by species and treatment regimen. PFOA induced a significant inhibition of catalase (CAT) activity at high concentrations with no changes of superoxide dismutase (SOD) or glutathione peroxidase (GPx) activities in the liver of male Japanese medaka[13]. Liu et al. reported significant induction of caspase-3,-8, -9 activities accompanied by increased levels of SOD, CAT activities and lipid peroxidation (LPO) level (measured by maleic dialdehyde, MDA) in PFOA treated hepatocytes of freshwater tilapia, whereas GPx activity was decreased[7]. PFOA affects the circulating sex steroid levels, increases hepatic fatty acyl-CoA oxidase activity, and increases oxidative damage[10,15]. Rare minnow exposed to PFOA for 28 d exhibits the suppression of gene related to fatty acid biosynthesis and mitochondrial fatty acid beta-oxidation[26]. Another study confirms that exposure of salmon to PFOA produce changes in mRNA expression for PPARs, ACOX1, oxidative stress responses and lipid β-oxidation, but these responses show marked organ differences[1].

The zebrafish embryo is an appropriate vertebrate model for investigating developmental toxicity of environmental contaminants[22,24]. This is mainly due to its easy maintenance, the short reproduction cycle and the high number of eggs per spawning, the rapid development, the transparency of the eggs and the well-documented and abundant biological and genetic information. Nevertheless, effect of PFOA induced oxidative damage in this valuable model fish remains largely unclear. Therefore, this study attempted to analyze the mechanism of PFOA-induced oxidative toxicity in zebrafish. The changes in the activities of antioxidative enzymes and lipid peroxidative product were measured. The effect of PFOA on apoptosis related gene expression (e.g., Bax and p53) were further assessed in a short-term zebrafish assay *in vivo*.

MATERIALS AND METHODS

Zebrafish maintenance and embryo exposure

Adult zebrafish were maintained at 28 ± 0.5°C in a 14/10 h light/dark cycle in a closed flow- through water. Tap water was treated to remove residual ammonia, chlorine and chloramines. The fish were fed with live artemia nauplii twice daily. Zebrafish embryos were obtained from spawning adults in groups of about 6 males and 6 females in tanks overnight. Mating, spawning, and fertilization took place within 30 minutes after light onset in the morning. Eggs were collected from spawn traps and washed with clean water. At 6 hours post fertilization (hpf) embryos were examined under a dissecting microscope, and those embryos that had developed normally and reached the blastula stage were selected for PFOA exposure. PFOA (96%) was obtained from Sigma Chemical Corporation (St Louis, MO). We observed the development of zebrafish embryos and replaced the exposure solutions every day, and the embryos were then grown up in an incubation chamber at 28 ± 0.5°C.

Activities of antioxidant enzymes

The exposure solution was removed and the embryos were rinsed gently with distilled water for three times. Then whole-body homogenates in 500 µl of cold phosphate buffered saline (PBS, pH=7.5) were immediately prepared and centrifuged at 12000 g for 15 min at 4°C. The supernatants were collected for various assays.

Catalase (CAT) and superoxide dismutase (SOD) activities were measured following the instructions of the commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). CAT activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption. One unit of CAT activity was defined as the amount of enzyme decomposing 1 µmol H_2O_2 in 1 s. SOD activity was evaluated by the inhibition rate of the superoxide radicals–dependent cytochrome C reduction. The result of this enzymatic assay was given in units of SOD activity per milligram of protein (U/mg), where 1 U of SOD was defined as the amount of sample causing 50% inhibition of cyto-

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chrome C reduction.

Lipid peroxidation and protein assay

Lipid peroxidation (measured as MDA) level was determined by the kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The MDA level was expressed as nanomoles per milligram protein. Protein concentrations were measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ).

Total RNA extraction and quantitative real-time PCR (QPCR)

Total RNA was extracted from 15 zebrafish embryos for each group using Trizol reagent (Invitrogen, California, USA) followed by the purification step of phenol-chloroform extraction according to the instructions of manufacturer. RNA integrity was assessed by standard denaturing agarose gel electrophoresis. The concentration of total RNA was measured based on the A260 value. Real-time PCR was performed with a ABI 7500 fast Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA) using SYBR Premix ExTaq™ (Takara Bio., Japan). The gene-specific primers were used as previously reported[12]. Each reaction was performed in three replicate samples. The expression level of each target gene was normalized to β-actin content. The melting curve was analyzed to differentiate the desired amplicons and the primer-dimers or DNA contaminants.

Statistical analysis

All results were presented as mean ± SEM. The differences were evaluated by one-way ANOVA followed by Tukey's test using SPSS software. A *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Activities of antioxidative enzymes

SOD, the endogenous scavenger, catalyzes the dismutation of the highly reactive superoxide anion to H_2O_2. In PFOA treated zebrafish embryos, SOD activities were significantly increased by 60, 110, 190 and 83% in the 1, 5, 10 and 15 mg L^{-1} exposure groups, respectively (Figure 1).

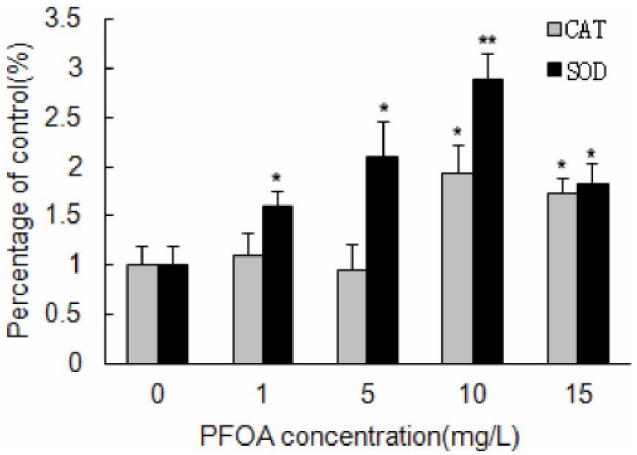

Figure 1 : Changes of SOD and CAT activities in zebrafish larvae exposed to different concentrations of PFOA. Values that were significantly different from the control were indicated by asterisks (one-way ANOVA, *$p < 0.05$; **$p < 0.01$). Values were the means of four replicate exposures and were presented as mean ± SEM.

CAT is responsible for the reduction of hydrogen peroxide and protection from the oxidation of unsaturated fatty acids in cell membrane. CAT activities were significantly increased by 93 and 73% in the 10 and 15 mg L^{-1} exposure groups, respectively. The CAT activities were slightly increased or decreased but not significantly changed in the 1 and 5 mg L^{-1} PFOA exposure groups (Figure 1).

Lipid peroxidation

MDA was indicative of lipid peroxidation. MDA contents were increased by 74% and 86% in the higher concentration treated groups (10 and 15 mg L^{-1}) compared with the control. The MDA contents were slightly increased but not significantly changed in the 1 and 5 mg L^{-1} PFOA exposure groups ($p>0.05$) (Figure 2).

Effects of PFOA exposure on mRNA expression of P53 and Bax

We measured gene expression of Bax and p53 by Q-PCR. The mRNA expression of Bax gene was significantly increased 1.6- and 1.8- fold in 5 and 10 mg L^{-1} PFOA exposure groups. The Bax transcription was also increased in the 1 and 15 mg L^{-1} PFOA exposure groups, but the change was not statistically significant ($p>0.05$) (Figure 3A).

p53, a critical gene involved in the regulation of apoptosis, was induced in PFOA treated animals compared with the control. p53 gene transcription was sig-

nificantly increased by 2.1- , 2.0- and 2.9- fold in the 5, 10 and 15 mg L^{-1} PFOA exposure groups, respectively. There was no significant difference in 1mg L^{-1} treatment group compared with the control (Figure 3B).

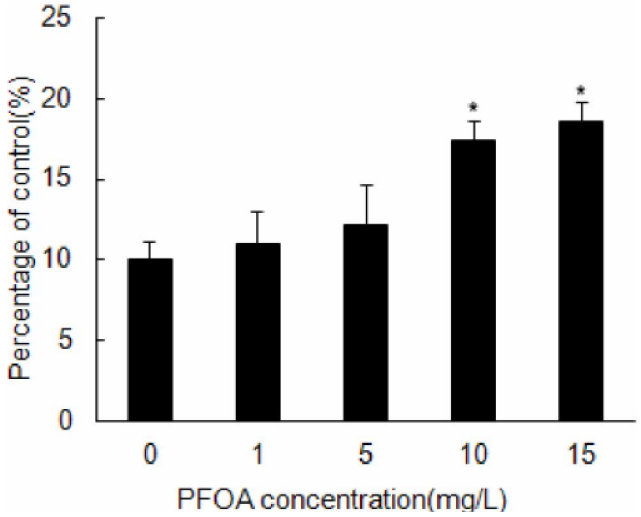

Figure 2 : Changes of MDA level in zebrafish larvae exposed to different concentrations of PFOA. Values that were significantly different from the control were indicated by asterisk (*$p < 0.05$). Values were the means of four replicate exposures and were presented as mean ± SEM.

DISCUSSION

The zebrafish develop rapidly and gastrulation begins at about 6 hpf. Spontaneous movements emerge and tail is detached from the yolk with heart beating at 24 hpf, and embryogenesis is essentially completed at 96 hpf, as most organs are formed and functioning by this time[8]. Thus at 6 hpf embryos were examined under a dissecting microscope, and those embryos that developed normally and reached the blastula stage were selected for PFOA exposure.

Fish embryonic development may be especially sensitive to toxicant-induced oxidative stress since even a 15–20% increase in ROS can tip progenitor cells into premature cell cycle arrest or differentiation[21]. Excessive rate of ROS formation as a result of exposure to environmental pollutants may exceed the antioxidant capacity and subsequently result to oxidative stress in organisms. Thus, organisms are able to adapt to ROS formation by increasing the expression of antioxidant enzymes, in addition to many other forms of defense and repair of oxidative damages[3]. The enzymes SOD and CAT play significant roles as antioxidants and their elevated expression and activity are indicative of oxidative stress. SOD is a metalloenzyme that catalyzes the dismutation of superoxide radicals ($.O_2^-$) to O_2 and H_2O_2, while CAT subsequently reduces the produced H_2O_2 to H_2O in the peroxisomes. In the present study, SOD and CAT activities were significantly changed. Alteration of these parameters indicated that oxidative stress-related process was involved in the PFOA-induced *in vivo* toxicity.

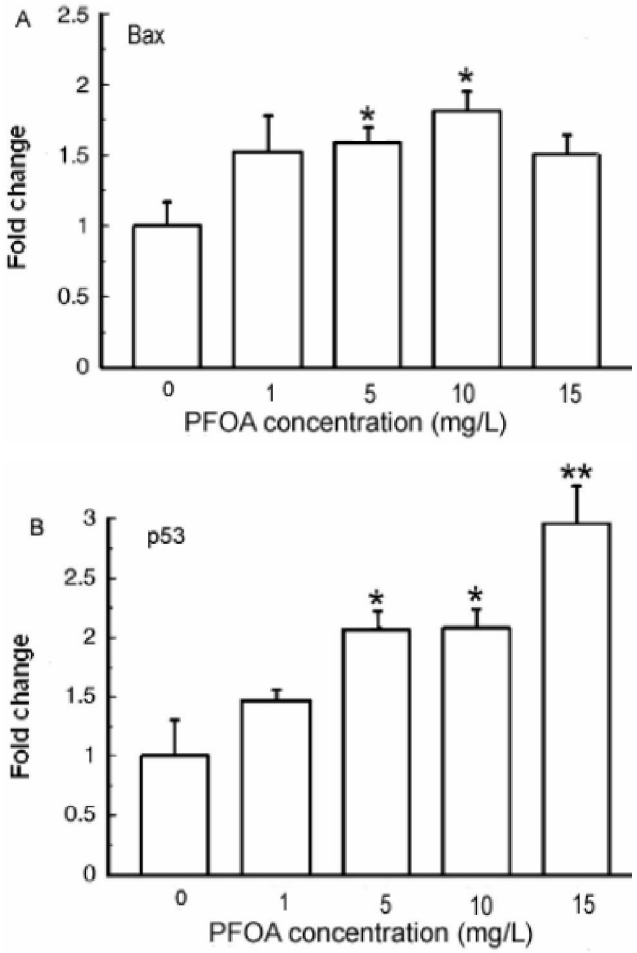

Figure 3 : Real-time quantitative PCR analyses of gene expression levels of p53 and Bax in the zebrafish larvae exposed to different concentrations of PFOA. Values that were significantly different from the control were indicated by asterisks (one-way ANOVA, *$p < 0.05$; **$p < 0.01$). Values were the means of three determinations on each of the three replicate exposures and were presented as mean ± SEM.

SOD activity was significantly increased presumably to counteract oxidative damage. However, higher concentration of PFOA (15 mg L^{-1}) appeared to alleviate SOD activity (Figure1). This was similar with the

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previous report that higher concentration of PFOA (30 mg L^{-1}) induced no significant change in SOD activity while lower concentration could increase SOD activity in cultured hepatocytes of freshwater tilapia[7]. This might be explained by the excess production of superoxide radicals, which, after their transformation to H_2O_2, caused an oxidation of the cysteine in the enzyme and deactivate SOD activity. Catalase serves as a primary defense enzyme against oxidative stress such as hydrogen peroxide generated from peroxisomal-oxidation of fatty acids[17]. The change of CAT activityin this study suggested that PFOA might induce oxidative stress through the alteration of cellular oxidative homeostasis.

Environmental stressors result in an imbalance between ROS production, and scavenging by endogenous antioxidants can directly or indirectly disturb physiological functions of many cellular macromolecules such as DNA, protein, and lipids and activate cellular stress-sensitive signaling pathways. Lipid peroxidation is commonly used as marker of oxidative stress response in aquatic animals, and has been shown to be significant contributors of reduced cell function under oxidative stress conditions[23]. In the present study, we observed a significant increase in a lipid peroxidative product (MDA) at higher exposure groups. The results suggested that PFOA exposure might affect cell membrane function and thus impair early development of zebrafish embryos. Notably, no discernable lipid peroxidation induction was observed in 1 and 5 mg L^{-1} exposure groups, while SOD changed dramatically at these concentration points, indicating the contribution of the antioxidant defense system.

Stress-induced apoptosis is thought to contribute to abnormal development during embryogenesis. The tumor suppressor gene p53 has been examined for its role in DNA damage-induced apoptosis that is chemically induced[24]. Typically, p53 is activated when DNA damage occurs or the cell is stressed. Most apoptotic signaling processes are related to alterations of apoptosis-related molecules, such as p53, Bcl-2/Bax, cytochrome c etc., which then trigger caspase activation via caspase-3 and subsequently induce cell apoptosis[18,20]. On the basis of results obtained in the present study, it might be hypothesized that, in embryos exposed to PFOA, ROS induced oxidative stress and caused damage to cell membrane (as evident from increased LPO level) and subsequently, the oxidative stimulus might trigger p53. It translocated to the nucleus, where it could induce proapoptotic gene expression, such as Bax, on the mitochondrial membrane, activated the downstream effector and initiated apoptosis.

In summary, our results clearly showed that PFOA could induce antioxidative responses in zebrafish embryos, resulting in oxidative damages. Further studies are needed to evaluate the toxic potential of this compound in fish models with the extended exposure time points and concentrations. Furthermore, the relationship between the protective role of certain gene function and developmental toxicity remains to be further investigated.

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